Applicants' Invention and its Advantages

The invention is drawn to methods of producing purified caveolae, the methods including an immunoisolation step of incubating a sample containing plasma membranes with an antibody that is specific for caveolin and which binds to oligomerized caveolin found aroungd intact caveolae (i.e., which binds to caveolin in its native state as an oligomeric structural cage surrounding intact caveolae). The invention is further drawn to purified caveolae prepared by specific methods as described above. The methods are simple and efficient means of producing purified caveolae which closely resemble caveolae in their native state (e.g., caveolae covered with the oligomeric structural cage of caveolin); the methods also minimize contamination and loss of molecules that dissociate from caveolae over time. Furthermore, the methods do not require perfusion of a tissue or coating of the plasma membranes with colloidal silica (described, for example, in US Patent 5,776,770), and thus allow a high level of flexibility of starting materials, as the methods can be used even for tissues or samples that cannot be perfused or coated with colloidal silica.

Rejection of Claims under 35 U.S.C. 102(b)

The Examiner rejected Claims 1-7, 11, 13-15 and 19-20 as being anticipated by Stan *et al.* (AS2), stating that Stan *et al.* describe subjecting fractions of plasma membranes to immunoisolation by polyclonal antibodies which "would inherently bind the oligomerized form of caveolae since they recognized the caveolae in its natural state, prior to being denatured (see figure 2)". Stan *et al.* describe a method of purification of caveolae which includes immunoisolation of caveolae on anti-caveolin coated magnetic beads (Figure 2 of Stan *et al.*). However, the antibody used in the immunoisolation of Stan *et al.* differs from the antibody described in the claimed methods.

Stan *et al.* specifically state that an antibody they had prepared themselves was used in all immunoisolation of caveolae (see page 598, second column, under the heading, "Antibody Characterization," where it is stated that "Our antibody was used exclusively for immunoisolation of caveolae on magnetic beads..."). This antibody, described as "anticaveolin-N" antibody, was separated from polyclonal sera raised in rabbits against synthetic peptides covalently coupled to keyhole limpet hemocyanin. "Anticaveolin-N" antibody was thus a fraction of a polyclonal antiserum, and not a monoclonal antibody. Stan *et al.* state that the synthetic peptides used the N-terminal residues 1-14 of chicken caveolin (see page 597, first

column, under the heading, "Antibody Production"). It should be noted, however, that the 16 N-terminal residues set forth by Stan *et al.* (MSGGKYUSDSEGHLYC) are not the N-terminal residues of chicken caveolin: they differ from the N-terminal residues of chicken caveolin that are set forth in the GenBank data base. A copy of each of the two entries pertaining to chicken caveolin is attached to this Amendment as Appendix I for the convenience of the Examiner. At most, 11 of the 16 residues set forth by Stan *et al.* are at the N-terminal region of chicken caveolin. The anticaveolin-N antibody did recognize caveolin, as indicated by the reduction and abrogation of the 22 kDa caveolin signal when antigen (N-terminal peptide having the specified residues) was added to a sample of antibody and caveolin or antibody and P1 fraction (page 598, second column, under "Antibody Characterization"). However, due to the difference between the synthetic peptide used and the actual sequence of the caveolin peptide, one of ordinary skill in the are would expect that the anticaveolin-N antibody would have a lower affinity to caveolin than other caveolin-specific antibodies.

The antibodies of Stan *et al.* differs from the antibody used in the methods of the intention. As indicated in the Specification, the representative antibody used in the methods of the invention is a monoclonal antibody that not only binds caveolin in its native state as an oligomeric structural cage surrounding intact caveolae, but also specifically binds the alpha-isoform of caveolin-1 via a specific epitope found in the N-terminal segment that is not present in the beta-isoform (p. 13, lines 13-18, citing Scherer *et al., J. Biol. Chem. 270*:16395-16401 (1995)). The Scherer *et al.* reference cited therein indicates that the alpha form (a slower migrating 24 kDa species) and the beta form (a faster migrating 21 kDa species) differ at their N-terminus, specifically residues 1-21. It appears that the 14 N-terminal residues used by Stan *et al.* were derived from the faster-moving beta form of caveolin, since the antibody recognizing the 14 N-terminal residues also recognized a 22 kDa caveolin (see page 598, second column, under "Antibody Characterization"). Thus, it appears that the antibody of the invention and the antibody of Stan *et al.* recognize different epitopes. Furthermore, as discussed above, the residues used by Stan *et al.* in the generation of the polyclonal antisera from which the fraction of "anticaveolin-N antibody" was derived, are not the exact N-terminal residues from chicken caveolin; thus, as indicated above, binding of the antibodies of Stan *et al.* would be expected to be of a lower affinity and less specific for caveolin than the representative monoclonal antibody used in the methods of the invention.

In addition, binding of the anticaveolin-N antibody to the N-terminal residues set forth in Stan *et al.* is not equivalent to binding of an antibody to the oligomerized form of caveolae. It is most unlikely that the N-terminal residues coupled to the keyhole limpet hemocyanin, as used by Stan *et al.*, would have been able to form an epitope that is present in the oligomerized caveolin structural cage that surrounds caveolae, particularly since the residues do not, in fact, match the N-terminal residues of chicken caveolin. Furthermore, one of ordinary skill in the art would not assume that an antibody which binds to caveolin would inherently also bind to caveolin in a native state as an oligomeric structure. For example, the difference between an antibody that binds caveolin in its native state as an oligomeric structure, as opposed to one that solely binds to caveolin protein *per se*, can be seen in the Specification in Example 2 (pp. 13-14). There, it is seen that antibodies specific for caveolin in its oligomeric state (CAV antibody) were able to bind caveolin expressed on purified silica-coated endothelial cell membranes rapidly and with a high affinity; in contrast, other antibodies (antibodies 2297 and Z034) that were specific to caveolin by Western analysis (and thus specific for caveolin in its monomeric state after denaturation during Western analysis) showed very little to no reactivity to caveolin in purified silica-coated endothelial cell membranes (P).

In view of these considerations, it is clear that the antibodies used by Stan *et al.* are not antibodies that bind to oligomerized caveolin as is used in the methods of the invention, as both the epitope and the specificity are different from that of the antibody used in the methods of the invention. Thus, the claimed invention is not anticipated by the teachings of Stan *et al.*

Rejection of Claims under 35 U.S.C. 103

The Examiner rejected Claims 8, 9, 12, 16, 17, 21, 22, 24 and 25 as being unpatentable over Stan *et al.*, stating that it was within the purview of one of ordinary skill in the art to be motivated to optimize antibody incubation times and that shearing is a well recognized method to disrupt membranes.

Incubation Time

The incubation time for the Stan *et al.* immunoisolation procedure was overnight (page 297, second column, first full paragraph) There is no teaching or suggestion in Stan *et al.* that any other incubation time for the immunoisolation would produce different results, or that an extremely short (e.g., less than 2 hours) incubation time would be appropriate or desirable.

While one of ordinary skill in the art might adjust incubation times when optimizing an experiment, optimization typically does not include a dramatic departure from a typical incubation period (such as a reduction from overnight to less than 2 hours). In fact, given the overnight length of incubation set forth in Stan *et al.*, it is likely that one of ordinary skill in the art would assume that a lengthy period of time would have been necessary for the immunoisolation step, and would not have used a dramatically shorter time such as that set forth in the claimed invention.

Applicants compared the products produced by lengthy (overnight) incubation and short (1 hour) incubation (see the Specification in Example 7 at page 19, lines 3-19). It was determined that the short incubation allowed the caveolae to retain signaling molecules normally found in the caveolae, whereas overnight incubation caused a substantial release of signaling molecules into solution. Caveolae obtained after a short incubation time therefore retained more of the signaling molecules normally present in caveolae than caveolae obtained after a long incubation time. Thus, Applicants have for the first time demonstrated a specific benefit to use of a short incubation time, as opposed to a long incubation time, during immunoisolation. In view of these considerations, the claimed invention is not obvious over the teachings of Stan *et al.*

Membrane Disruption

Stan *et al.* describe the use of sonication as a membrane disruption method (page 597, first column, under "Purification of PVs" "Step III (Sonication)"). There is no teaching or suggestion in Stan *et al.* that any other membrane disruption method would produce different results upon immunoisolation of caveolae.

Applicants compared the fractions produced by sonication and by shearing (see the Specification in Examples 3 and 4, page 14, line 15, through page 16, line 19). It was determined that the fraction produced by sonication (the PC fraction) differed from the fraction produced by shearing (the V fraction). For example, the protein profiles indicated that sonication appeared to release additional low density vesicles into the PC fraction, that were not found in the V fraction (page 15, lines 4-10). Furthermore, the caveolin in the shearing fraction (V) was nearly all accessible and able to interact with immuno-beads during immunoisolation, resulting in a quantitative isolation of nearly all of the starting material. In contrast, when the sonication fraction (PC) was used, a significant amount of the caveolin was inaccessible to immunoisolation

(page 16, lines 5-9). Use of shearing therefore allowed isolation of more caveolae than would be obtained when using sonication. Thus, Applicants have for the first time demonstrated a specific benefit to use of shearing, rather than sonication, as a membrane disruption method prior to immunoisolation. In view of these considerations, the claimed invention is not obvious over the teachings of Stan *et al.*

CONCLUSION

In view of the discussion presented above, the claims are in condition for allowance. Applicants respectfully request that the Examiner reconsider and withdraw all rejections.

If the Examiner believes that a telephone conversation would expedite prosecution, the Examiner is invited to contact Elizabeth W. Mata at (915) 845-3558. If Elizabeth W. Mata cannot be reached, the Examiner is invited to contact Doreen Hogle at (781) 861-6240.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

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Lexington, Massachusetts 02421-4799
Dated: October 3, 2000

Appendix I

NCBI Sequence Viewer

PubMed Nucleotide Protein Genome Structure Popset

Search PubMed Protein Nucleotide Structure Genome PopSet OMIM for

Limits Index History Clipboard

ASN.1 FASTA GenPept Graphics XML Default View as HTML Plain Text
Hide Brief and LinkBar

1: GI = "422672" [GenPept] caveolin - chickenPubMed, Related Sequences, Taxonomy

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DEFINITION caveolin - chicken.
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VERSION A46424 GI:422672
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PIR dates: 21-Sep-1993 #sequence_revision 18-Nov-1994 #text_change 18-Nov-1994.
KEYWORDS .
SOURCE chicken.
ORGANISM Gallus gallus
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Archosauria; Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.
REFERENCE 1 (residues 1 to 200)
AUTHORS Glenney,J.R. Jr. and Soppet,D.
TITLE Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus-transformed fibroblasts
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 89 (21), 10517-10521 (1992)

MEDLINE 93066270
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NCBI Sequence Viewer

PubMed Nucleotide Protein Genome Structure Popset

Search PubMed Protein Nucleotide Structure Genome PopSet OMIM for

Limits Index History Clipboard

ASN.1 FASTA GenPept Graphics XML Default View as HTML Plain Text
Hide Brief and LinkBar

1: GI = "543937" [GenPept] CAVEOLIN-1PubMed, Related Sequences, Taxonomy

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PID g543937
VERSION P35431 GI:543937
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created: Jun 1, 1994.
sequence updated: Jun 1, 1994.
annotation updated: Jul 15, 1998.
xrefs: gi: 211426
xrefs (non-sequence databases): PFAM PF01146, PROSITE PS01210
KEYWORDS Transmembrane; Lipoprotein; Palmitate; Alternative initiation;
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SOURCE chicken.
ORGANISM Gallus gallus
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Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.
REFERENCE 1 (residues 1 to 178)
AUTHORS Glenney,J.R. Jr. and Soppet,D.
TITLE Sequence and expression of caveolin, a protein component of
caveolae plasma membrane domains phosphorylated on tyrosine in Rous
sarcoma virus-transformed fibroblasts

JOURNAL Proc. Natl. Acad. Sci. U.S.A. 89 (21), 10517-10521 (1992)
MEDLINE 93066270
REMARK SEQUENCE FROM N.A.
COMMENT ---
This SWISS-PROT entry is copyright. It is produced through a
collaboration between the Swiss Institute of Bioinformatics and
the EMBL outstation - the European Bioinformatics Institute.
The original entry is available from http://www.expasy.ch/sprot
and http://www.ebi.ac.uk/sprot
---.
[FUNCTION] MAY ACT AS A SCAFFOLDING PROTEIN WITHIN CAVEOLAR
MEMBRANES. INTERACTS DIRECTLY WITH G-PROTEIN ALPHA SUBUNITS
AND CAN
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[SUBUNIT] HOMOOLIGOMER (BY SIMILARITY).
[SUBCELLULAR LOCATION] MEMBRANE PROTEIN OF CAVEOLAE.
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121 ilsflhiwav vpcirsylie iqcisrvysi cihtfcdplf eamgkvfssi ratvrkei
//